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Role of Ca^{2+} and Ethanol in The Process of Flocculation

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In addition to the two pools of glycogen, cytoplasmic and cell wall bound insoluble glycogen, the third pool of glycogen at the cell surface was confirmed by amyloglucosidase treatment in yeast cell. Amyloglucosidase removes the cell surface α-glucans so that the yeast cells lose their capacity to form aggregates in the presence of concanavalin-A. Cells grown in a medium containing Ca^{2+} ions exhibit higher amount of cell surface α-glucans. Addition of both Ca^{2+} and ethanol to yeast culture medium results in increased both insoluble glycogen content and surface α-glucans which play an important role in the flocculation process. Hence both Ca^{2+} and ethanol enhance flocculation in yeast cells. The flocculance level correlates with the level of cell surface α-glucans and the rate of flocculation of yeast. Highly flocculant yeast cells have higher level of cell surface α-glucans and rapid setting behaviour.

Key Words: Flocculation, α-Glucans, Ethanol, Calcium, Yeast, *S. cerevisiae*.

INTRODUCTION

The surface of yeast cells plays a major role in aggregation, flocculation and adhesion, which are important for the production of bright beer. During flocculation, aggregating solitary yeast cells form aggregates of thousands of cells. Flocculation depends upon cell surface properties such as electrical charge, hydrophobicity and surface chemical composition and cultural conditions like pH, temperature and medium composition[1]. The thick rigid cell wall of yeast consists of 80 % carbohydrate in the form of α-glucan, β-glucan, mannan and chitin. Calcium ions play an important role in the flocculation over a broad pH range[2]. The most generally accepted mechanism of flocculation involves lectins that recognize and bind to carbohydrate such as glucose and mannose residues present on adjacent cells. Lectin mediated flocculation mechanism requires Ca^{2+} ions to maintain their active conformation[3]. Calcium bridging hypothesis was suggested[4,5] according to which Ca^{2+} ions are required for mutual adhesion of cells by forming salt bridges between their surface carboxyl groups. Such salt bridges are supported by hydrogen bonding between hydroxyl groups of cell wall carbohydrate such as mannan. Flocculation varies with changes in acid soluble glycogen content[6] of yeast. Flocculation is an inheritable characteristic of yeast cells that involves FLO genes like FLO1, FLO2,

FLO4, FLO5 and FLO8. Flocculation level in yeast is determined by FLOP, a flocculation protein present at the cell surface[7]. Cell surface hydrophobicity, charge and zymolectin density are important determinants of flocculation in *S. cerevisiae*[8]. Due to different surface properties like chemical composition, surface hydrophobicity and electrical properties that affect cell flocculation and sedimentation, brewery yeast cells are classified as a top and bottom fermenting strains. Top and bottom yeasts flocculate by different mechanisms[9]. Flocculation in bottom fermenting yeast is mediated by lectins and is enhanced by Ca^{2+} ions[5,10,11], while top fermenting yeast cells require ethanol to enhance their flocculation. Calcium ion plays an important role in the flocculation and increases ethanol tolerance in yeast. Externally added ethanol plays an osmoprotective role for yeast which is reflected in the variation of cell wall glycogen content and increases the amount of cell surface glycogen contributing the level of flocculation. This study investigates the relationship between cell surface a glucan, cell wall glycogen and flocculation in *S. cerevisiae*. Individual cell ageing in brewing strains may influence fermentation performance in yeast cells[12].

EXPERIMENTAL

Aspergillus niger amyloglucosidase (glucoamylase) from Sigma Chemical Company, U.S.A. Glucose Oxidase kit from Bio. Lab. Peptone, yeast extract and malt extract from Difco Laboratories, Detroit, U.S.A.

Alcohol estimation was carried out by ceric ammonium nitrate method[13]. Total polysaccharide was determined by the phenol sulphuric acid method[14]. Glucose was measured by glucose oxidase method[15].

Isolation of glycogen: Glycogen was isolated from yeast cells by alkali digestion method[16] that yields reddish brown coloured yeast digest. The yeast digest was separated into supernatant and residual fractions containing soluble and insoluble glycogen by centrifugation at 8000 rpm for 20 min.

Estimation of glycogen: 1 mL volumes of the digest supernatant and residual sediment were incubated with 1.2 mL solution containing sodium acetate buffer, pH 4.2, amyloglucosidase (2 I.U.) and α-amylase (1 I.U.) at 37 ºC for 1 h. Glucose liberated in the reaction mixture was determined. The amount of glucose in the supernatant and residual sediment represents soluble and insoluble glycogen respectively.

Determination of surface α-glucan: Yeast cells were fixed by using 1.5 % glutaraldehyde. 1 g yeast suspended in 4 mL of sodium acetate buffer (100 mM, pH 4.2) was then treated with 0.3 mL of amyloglucosidase at 37 ºC for 1 h. The supernatant obtained after this treatment was used for determination of surface a glucan content by the glucose oxidase method.

Determination of flocculence level in yeast cells: Yeast cells harvested from culture in YPD medium were used to determine their flocculation rates. 0.7 g cells aliquots of *S. cerevisiae* with strain Nos. 3441, 109 and MTCC-12 were weighed accurately in glass centrifuge tubes and then suspended in 5 mL of 50 mM sodium

acetate buffer (pH 4.2) containing 20 mmol/L $CaCl_2$. The cell suspensions were agitated properly for uniform distribution of cells in the solution, held standby for 10 min and then centrifuged (6000 rpm, 20 min). 2 mL of supernatant was carefully taken from each tube and the optical density was measured at 530 nm.

Treatment of cells with concanavalin-A: 0.5 g yeast cells (wet weight) were suspended in 4 mL 100 mmol/L acetate buffer, pH 4.2. Then 1 mL of concanavalin-A (Con-A) (0.2 mg/mL) solution was added. Resultant cell suspension was agitated, allowed to stand for 4 h and then examined under the microscope to observe formation of cell aggregates.

Effect of amyloglucosidase treatment on aggregation of yeast cells: 0.5 g yeast (wet weight) was suspended in 4 mL of 100 mmol/L acetate buffer, pH 4.2. Then 0.5 mL of amyloglucosidase (3 I.U.) was added and the cell suspension was incubated at 37 ºC for overnight. Con-A was then used to test the aggregation these cells.

Organisms and media: *S. cerevisiae* strains 3300 (bottom strain), 109 (top strain) and 3441 (top strain) were purchased from National Chemical Laboratory, Pune, India. Bottom flocculating strain (MTCC-12) was from the Institute of Microbial Technology, Chandigarh. All strains were maintained on YPD at 4 ºC.

Media composition

A) Effect of $CaCl_2$ on yeast glycogen and ethanol content: (*S. cerevisiae* 3300)

YPG: (3 g/L yeast extract, 5 g/L peptone, 80 g/L glucose).

YPD-LCa: (3 g/L yeast extract, 5 g/L peptone, 80 g/L glucose, 0.8 g/L $CaCl_2$).

YPD-HCa: (3 g/L yeast extract, 5 g/L peptone, 80 g/L glucose, 80 g/L $CaCl_2$).

B) Effect of $CaCl_2$ and ethanol on yeast glycogen : (*S. cerevisiae* 3300)

YPDE: (3 g/L yeast extract, 5 g/L peptone, 80 g/L glucose, 8 % v/v ethanol).

YPD-LCaE: (3 g/L yeast extract, 5 g/L peptone, 80 g/L glucose, 0.8 g/L $CaCl_2$, 8 % v/v ethanol).

YPD-HCaE: (3 g/L yeast extract, 5 g/L peptone, 80 g/L glucose, 80 g/L $CaCl_2$, 8 % v/v ethanol).

C) Flocculation study of yeast strains

(*S. cerevisiae* 3441)-YPD-I: (3 g/L yeast extract, 5 g/L peptone, 80 g/L glucose).

(*S. cerevisiae* 109)-YPD-II: (3 g/L yeast extract, 5 g/L peptone, 80 g/L glucose).

(*S. cerevisiae* MTCC-12): YPD-III (3 g/L yeast extract, 5 g/L peptone, 80 g/L glucose).

D) Aggregation of yeast cells: YP (3 g/L yeast extract, 5 g/L peptone).

The flasks with media YPD, YPD-LCa, YPD-HCa, YPD-E, YPD-LCaE and YPD-HCaE as well as the sugar free YP medium used to test yeast aggregation were autoclaved and inoculated. 8 % (v/v) ethanol was simultaneously added to the flasks containing YPD-E, YPD-LCaE and YPD-HCaE media. After 48 h of fermentation, yeast cells were harvested from the flasks, all variants except YP culture were subjected to alkali digestion. The digest supernatant and pellet were used to determine soluble and insoluble glycogen, respectively.

1 g of cells harvested from each flask was also treated with amyloglucosidase to determine the surface α-glucan content. Culture filtrates from YPD, YPD-LCa, YPD-HCa media were used to estimate ethanol content by ceric ammonium nitrate method. Yeast cells of all 3 strains (3441, 109 and MTCC-12) harvested from YPD medium were used to determine the flocculence level by measuring their glycogen, surface α-glucan and protein content as well as their rates of flocculation. Yeast cells grown in sugar free YP medium were used to test the aggregation property in the presence of Con-A.

RESULTS AND DISCUSSION

Effect of $CaCl_2$: The effect of $CaCl_2$ on glycogen and carbohydrate content of yeast cells is shown in Figs. 1 and 2. The amount of insoluble glycogen (8.3 mg) and carbohydrate (21.2 mg) in yeast cells from medium YPD-LCa containing 0.8 g/L $CaCl_2$ was similar as in cells grown in YPD medium without $CaCl_2$ (8.2 mg glycogen and 22.0 mg carbohydrate). Cells grown in the medium YPD-HCa containing excess of $CaCl_2$ (8 g/L) exhibit lower insoluble glycogen (5.1 mg) and carbohydrate (17.1 mg) content. However, cells from both YPD-LCa and YPD-HCa media exhibited the higher amount of surface a glucan and slightly higher ethanol yield on addition to $CaCl_2$ the media (Table-1).

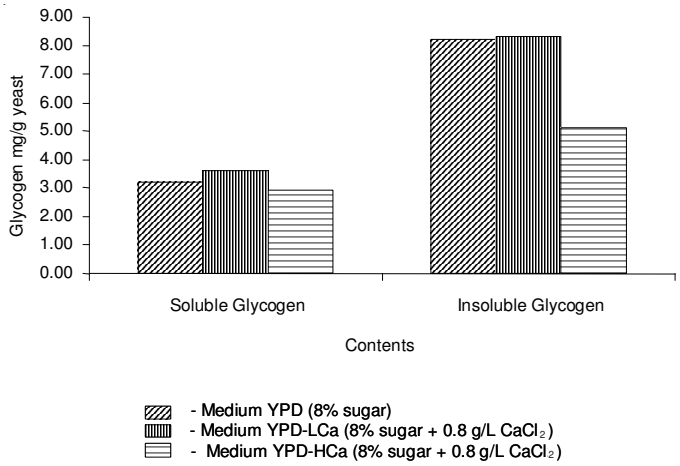

Fig. 1. Effect of $CaCl_2$ on yeast glycogen content (*S. cerevisiae* 3300)

TABLE-1
EFFECT OF $CaCl_2$ ON ETHANOL YIELD AND SURFACE α-GLUCAN CONTENT OF YEAST CELLS (*S. cerevisiae* 3300)

Media	Ethanol (%, v/v)	Surface α-glucan (μg/g) wet wt.	Media	Surface α-glucan (μg/g) yeast cell
YPD	3.3	259	YPD-E	259
YPD-LCa	3.8	345	YPD-LCaE	471
YPD-HCa	3.7	406	YPD-HCaE	438

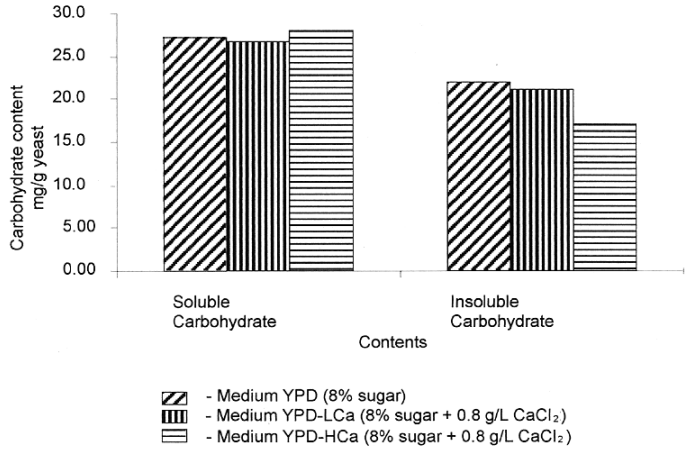

Fig. 2. Effect $CaCl_2$ on yeast carbohydrate content (*S. cerevisiae* 3300)

Effect of $CaCl_2$ and ethanol: Figs. 3 and 4 show the combined effect of $CaCl_2$ and ethanol on glycogen and carbohydrate content of yeast cells. Yeast cells from medium YPD-LCaE containing 0.8 g/L $CaCl_2$ and 8 % (v/v) ethanol display higher amount of insoluble glycogen (18 mg) and carbohydrate (34.6 mg) than cells grown in YPD medium while cells from the medium YPD-HCaE containing 8 % ethanol and excess of $CaCl_2$ exhibit lower insoluble glycogen (7.2 mg) and carbohydrate (20.1 mg) content. Cells from media YPD-LCaE and YPD-HCaE displayed a higher amount of surface a glucan (471 and 438 μg) respectively as a result of combined effect of $CaCl_2$ and ethanol (Table-1). In yeast cells grown in the presence as well as in absence of $CaCl_2$ and ethanol, soluble glycogen and carbohydrate content exhibit fewer variations.

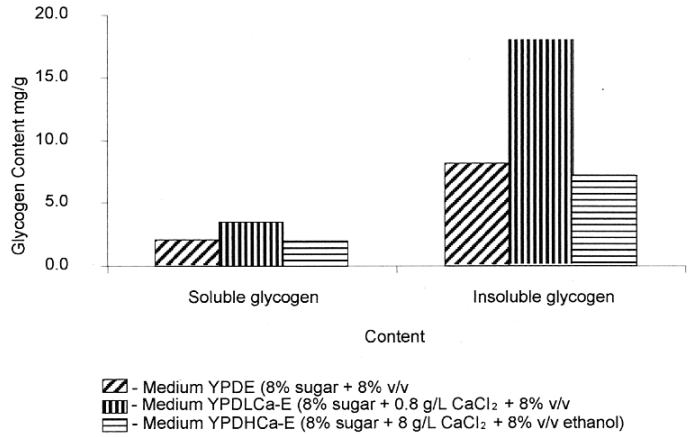

Fig. 3. Effect of $CaCl_2$ on yeast glycogen content (*S. cerevisiae* 3300)

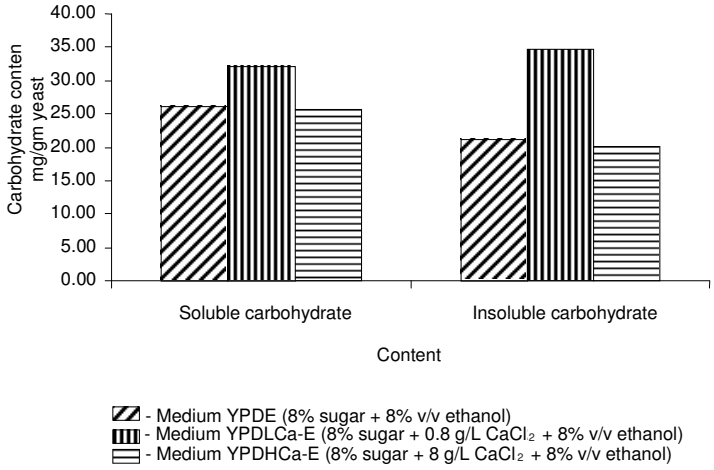

Fig. 4. Effect of $CaCl_2$ and ethanol on yeast carbohydrate content (*S. cerevisiae* 3300)

Yeast flocculation: Flocculation was studied using top (3441, 109) and bottom (MTCC-12) fermenting yeast strains. The degree of flocculance was determined on the basis of cell surface a glucan level and the rate of yeast flocculation. Table-2 showed that top flocculant strain 3441 had a higher amount of insoluble glycogen than bottom strains 109 and MTCC-12. However the percentage of surface a glucan in relation to total insoluble glycogen content was higher in MTCC-12 strain than that observed in 109 and 3441 strains. Flocculation rates of yeast strains (Table-2) indicate that the optical density of supernatant solution from cell suspensions (measured after 15 min) increase in *S. cerevisiae* 3441, *S. cerevisiae* 109, *S. cerevisiae* MTCC 12 in descending manner. Thus yeast cells of strain MTCC-12 settle down faster than cells of 109 and 3441 strains, respectively.

TABLE-2
AMOUNT OF GLYCOGEN, SURFACE α-GLUCAN AND PERCENTAGE OF α-GLUCAN IN YEAST CELLS

Strain	Insoluble glycogen (mg/g) wet wt.	Surface α-glucan (μg/g) wet wt.	α-Glucan to insoluble glycogen (%)	Surface protein (mg/g) wet wt.	(OD) at 530 nm
3441	39.00	1452	8.5	2.5	0.95
109	1.75	492	10.8	3.5	0.75
MTCC-12	14.00	682	12.8	4.0	0.21

Aggregation of yeast cells: Yeast cells grown in YP medium, treated with Con-A (Fig. 5B) and cells in presence of 8 % ethanol (Fig. 5C) aggregated to form larger flocs as compared to control (Fig. 5A), while the cells treated with amyloglucosidase exhibited reduced capacity to settle down and did not aggregate even in the presence of Con-A.

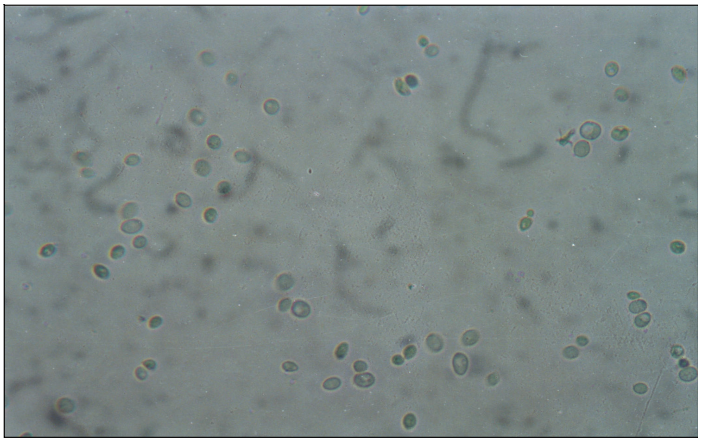

Fig. 5A. Control cell suspension (magnification 45 X)

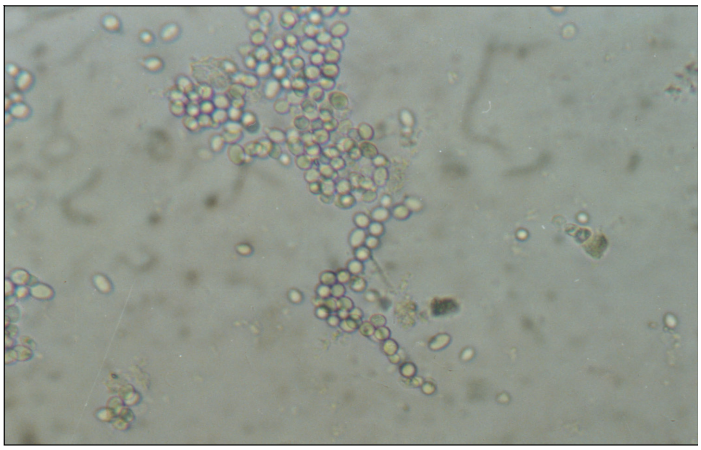

Fig. 5B. Cells propagated in the presence of concanavalin-A

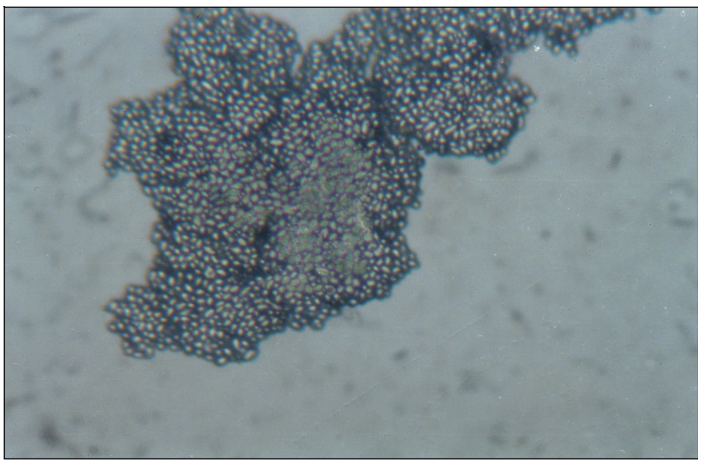

Fig. 5C. Cells propagated in the presence of 8 % (v/v)

Conclusion

There exists a positive correlation between the levels of ethanol, calcium and the α-β glucan complex referred to as insoluble glycogens. Both ethanol tolerance and glycogen content of yeast cells were increased in the presence of calcium. A combination of $CaCl_2$ and ethanol also displays higher amount of surface α-glucan (Table-1). Yeast glycogen is α-glucan having α (1, 4) and α (1, 6) linkages and it serves as a major storage carbohydrate. Glycogen synthesis and degradation in yeast is mediated by same set of enzymes as in mammals like glycogen synthase and glycogen phosphorylase involving regulatory c-AMP dependent cascade mechanism. Calcium which serves as a secondary messenger in eukaryotic cells plays an important role in yeast glycogen metabolism. This is reflected in increased glycogen synthesis and a higher amount of cell surface α-glucan in yeast cells grown in the presence of ethanol and Ca^{2+} ions. Ca^{2+} ions are involved in ion-water electrostatic interactions and play an important role in the lectin mediated flocculation mechanism by maintaining these lectins in active conformation. The fact that yeast cells grown in the presence of ethanol and Ca^{2+} ions exhibit an increase in surface α-glucan content that play an important role in cell aggragation indicates that flocculation is enhanced by both ethanol and Ca^{2+} ions, which must have correlation with cell surface α-glucans. Table-2 shows that highly flocculant yeast cells (MTCC-12) have a higher percentage of cell suface α-glucans in the total insoluble glycogen. Flocculation involving formation of aggregates enables the yeast cells to settle down rapidly. The rate of flocculation depends upon the concentration of cell surface α-glucans. Con-A enhances cell aggregation and flocculation obviously by binding to α-glucans. This role of α-glucans at the cell surface was confirmed by the treatment with amyloglucosidase, which specifically cleaves α (1, 4) and α (1, 6) bonds in glycogen type polysaccharides of yeast. Enzymatic treatment removes most of the cell surface α-glucans and resultant cells form poor aggregates and showed reduced capacity of flocculation.

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